The isolation and identification of new microalgal strains producing oil and carotenoid simultaneously with biofuel potential

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HIGHLIGHTS

• Four algal isolates from diverse habitats showed potential to produce multiple products.
• Functional variations for lipid and carotenoids production between isolates reported.
• Major fatty acids were palmitic, stearic, oleic, linoleic, and linolenic acid.
• Scenedesmus bijugus showed highest biomass productivity and multiple products potential.

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ABSTRACT

Taxonomy and phylogeny of twenty two microalgal isolates were examined using both universal and newly designed molecular primers. Among the isolates, Scenedesmus bijugus, Coelastrella sp., Auxenochlorella protothecoides, and Chlorella sp. were particularly promising in terms of producing lipids as measured by fatty acid methyl esters (FAME) analysis and significant concentration of carotenoids. A comparative experiment showed that S. bijugus and Chlorella sp. were the most promising candidates (L−1 d−1, with biomass) 174.77 ± 6.75, 169.81 ± 5.22 mg, lipids 40.14 ± 3.31, 39.72 ± 3.89 mg, lutein 0.47, 0.36 mg, and astaxanthin 0.27, 0.18 mg respectively. The fatty acids produced by these microalgal isolates were mainly palmitic, stearic, oleic, linoleic, and linolenic acid. The freshwater microalgal isolate S. bijugus be the most suitable isolate for producing biodiesel and carotenoids, due to high productivity of biomass, lipids, metabolites, and its suitable fatty acid profile.

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1. Introduction

Microalgae, widely distributed, and with a longer evolutionary history than terrestrial plants, show a rich diversity among their more than 200,000 species (Guiry and Guiry, 2014). However, 30,000 species have been studied but so far not fully exploited (Mata et al., 2010). Bioprospecting of microalgae has been carried out from ecologically diverse habitats such as the deep seas (Boeuf and Kornprobst, 2009) and polluted waters (Sterrenburg et al., 2007). Such diverse habitats may harbour distinct isolates with unique properties and multiple applications. During the past few years, microalgae have been extensively explored for biofuel and for bioactive compounds. In recent years, unique strains have been isolated from a range of habitats in the tropics including both aquatic (lakes, streams, and backwaters) and terrestrial habitats. Such extremes as very high temperatures and prolonged exposure to intense light found in the tropics have conferred on the extremophiles some distinctive physiological properties (Gouveia et al., 2009). Since the early 1970s, bioprospecting of microalgae (such as green algae, cyanobacteria, and diatoms) has been attempted for various uses, and their ability to consume carbon dioxide during photosynthesis and to act as a sustainable source of biofuels has led to increasing attention in recent years (Jones and Mayfield, 2011). Both lipid and biomass content are equally important for achieving higher lipid productivity (Yen et al., 2013). Microalgal lipids are divided in two main categories, those used as biofuel (with 14–20 carbon chains) and those used as food (containing 20 carbon chains) because they are rich in essential nutrients that are not synthesized by higher eukaryotic organisms (Jacob-Lopes et al., 2015). Deriving multiple products such as lipids and high-value by-products from the same biomass in one growth cycle is one way to make process economically sustainable (Nobre et al., 2013). Biologically active compounds derived from
microalgae have attracted increasing industrial attention (De Morais et al., 2015). The diverse gene pool of microalgae still remains to be fully explored for production of bioactive compounds, particularly using the biorefinery approach. So far, the market for carotenoids is primarily based on β-carotene and astaxanthin whereas lutein have not been similarly exploited. The importance of lutein and astaxanthin from microalgae has grown significantly in recent years. Lutein is a carotenoid that plays a role in the maintenance of healthy eyes, skin, and cardiovascular health, and is therefore used as a food additive in the preparation of many foods, including infant formulas and supplements. Astaxanthin, on the other hand, is a potent antioxidant that has been shown to have anti-inflammatory properties and may help prevent some degenerative diseases of the eye; it is used as a food additive in the pigmentation of salmonids, ornamental fish, and in the poultry industry (Lorenz and Cysewski, 2000). The market for these bioactive compounds continues to expand. Thus, new microalgal species that can produce these or other useful compounds is a continuing area of research.

It is against this background that the present study sought to compare twenty-two microalgal isolates for identifying the strains most suitable for biofuel and carotenoids production. The approach was to isolate a number of microalgae from a range of diverse habitats and to characterise the isolates using 18S rDNA analysis. The selection criteria were the ability to produce (1) lipids with fatty acid methyl ester (FAME) profiles suitable for biofuels and (2) carotenoids, which are valuable compounds that can be readily separated from oils and would potentially offset the cost of biofuel production.

2. Materials and methods

2.1. Collection, isolation, and purification of microalgal isolates

A total of two hundred isolates were collected from different habitats – aquatic and terrestrial rocky – in Asia, North America, and the Middle East (Table 1). The aquatic habitats included freshwater, chemically polluted river water, and backwater. Twenty-two microalgal isolates from Chlorellaceae and Scenedesmaceae were selected based on their molecular taxonomy and assessed for their potential to produce biofuel and carotenoids. The isolates from these two families were priorities for selection because under low light conditions, in Chlorella sp. lipid production is increased (Takeshita et al., 2014) whereas Scenedesmaceae family members yield both lipid and carotenoids (Peng et al., 2012). For photoautotrophic cultivation, primary stock cultures from different habitats were incubated up to the late exponential phase in full-strength Bold’s basal medium (BBM) (Andersen et al., 2005) in 100 mL Erlenmeyer flasks at 25 °C with orbital shaking at 150 rpm and under 16 h of fluorescent white light (120 μE m⁻² s⁻¹) alternating with 8 h of darkness and with constant shaking (150 rpm). Cell suspensions of equal strength of the isolates were also grown in experimental tubes containing 70 mL of the medium in Multi-Cultivator (MC) 1000-OD (Photon Systems Instruments, Drasov, Czech Republic). To ensure that cell counts for the comparative growth experiments the same initially, the number of cells was counted using a Neubauer haemocytometer (Rohm Instruments, Nashik, Maharashtra, India). The initial optical density (OD) of the cell suspension with a concentration of 3 × 10⁵ cells/mL was recorded. All the tested isolates were maintained at an illumination intensity of 120 μE m⁻² s⁻¹ as a standard growth condition. The source of illumination was placed 2.0 cm away from the external wall of the vessel, the internal diameter of which was 15 mm at 25 °C. Cultures were aerated with plain air at rate of 0.12 mL/min throughout the experiment.

2.2. Culturing of microalgal isolates

The twenty-two isolates were grown in 250 mL Erlenmeyer flasks containing 100 mL BBM medium and incubated for nine days at 25 °C under 16 h of fluorescent white light (120 μE m⁻² s⁻¹) alternating with 8 h of darkness and with constant shaking (150 rpm). Cell suspensions of equal strength of the isolates were also grown in experimental tubes containing 70 mL of the medium in Multi-Cultivator (MC) 1000-OD (Photon Systems Instruments, Drasov, Czech Republic). To ensure that cell counts for the comparative growth experiments the same initially, the number of cells was counted using a Neubauer haemocytometer (Rohm Instruments, Nashik, Maharashtra, India). The initial optical density (OD) of the cell suspension with a concentration of 3 × 10⁵ cells/mL was recorded. All the tested isolates were maintained at an illumination intensity of 120 μE m⁻² s⁻¹ as a standard growth condition. The source of illumination was placed 2.0 cm away from the external wall of the vessel, the internal diameter of which was 15 mm at 25 °C. Cultures were aerated with plain air at rate of 0.12 mL/min throughout the experiment.

2.3. Characterisation of isolates by scanning electron microscopy

For examining the cultures under a scanning electron microscope, 2 mL of the fully grown culture was centrifuged at 6000 rpm for 5 min. Primary fixation of the algal cells was performed by incubating them in 2.5% glutaraldehyde at room temperature in the dark for at least 4 h followed by secondary fixation in 1% osmium tetroxide overnight at 4 °C. The algal cells were dehydrated gradually in increasing ethanol series (10%, 30%, 50%, 70%, 90%, and 100%), critical point dried, sputter coated with gold palladium using a polaron SC7640 auto/manual high resolution sputter coater (Quorum Technologies, Lewes, East Sussex, UK) and examined under a FEI Quanta 200 scanning electron microscope (FEI, Hillsboro, Oregon, USA) at 10 kV.

<table>
<thead>
<tr>
<th>Region and country of collection</th>
<th>Number of isolates</th>
<th>Sampling month and year</th>
<th>Habitat</th>
<th>Latitude</th>
<th>Longitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>Middle East, Qatar</td>
<td>15</td>
<td>August 2012</td>
<td>Backwater</td>
<td>25° 17’ 7” N</td>
<td>51° 31’ 51” E</td>
</tr>
<tr>
<td>North America, St. Louis, USA</td>
<td>8</td>
<td>November 2012</td>
<td>Freshwater</td>
<td>38° 47’ 51” N</td>
<td>90° 47’ 8” W</td>
</tr>
<tr>
<td>Asia, Hussain Sagar, Hyderabad, India</td>
<td>8</td>
<td>August 2012</td>
<td>Freshwater</td>
<td>7° 25’ 25” N</td>
<td>78° 28’ 25.7” E</td>
</tr>
<tr>
<td>Asia, Rocky beach, Goa, India</td>
<td>7</td>
<td>May 2012</td>
<td>Backwater terrestrial, rocky</td>
<td>15° 17’ 57” N</td>
<td>74° 7’ 26” E</td>
</tr>
<tr>
<td>North America, Tree bark, St. Louis, USA</td>
<td>6</td>
<td>December 2012</td>
<td>Backwater terrestrial, rocky</td>
<td>38° 37’ 37” N</td>
<td>90° 11’ 57” W</td>
</tr>
<tr>
<td>Asia, Chennai, Tamil Nadu, India</td>
<td>25</td>
<td>June 2012</td>
<td>Backwater</td>
<td>11° 7’ 37” N</td>
<td>78° 39’ 24” E</td>
</tr>
<tr>
<td>Asia, Vishakhapatnam river, Andhra Pradesh, India</td>
<td>30</td>
<td>July 2012</td>
<td>Freshwater</td>
<td>41° 42’ 44” N</td>
<td>81° 15’ 14” W</td>
</tr>
<tr>
<td>Asia, Pangong lake, Ladakh, Jammu and Kashmir, India</td>
<td>6</td>
<td>April 2012</td>
<td>Freshwater</td>
<td>33° 44’ 16” N</td>
<td>79° 0’ 27” E</td>
</tr>
<tr>
<td>Asia, Mangalore, south-western Karnataka, India</td>
<td>20</td>
<td>June 2012</td>
<td>Backwater</td>
<td>12° 54’ 50” N</td>
<td>74° 51’ 21” E</td>
</tr>
<tr>
<td>Asia, Yamuna river, river, India</td>
<td>60</td>
<td>July 2012</td>
<td>Chemically polluted river water</td>
<td>28° 36’ 50” N</td>
<td>77° 12’ 32” E</td>
</tr>
<tr>
<td>Asia, Yamuna river, Delhi, India</td>
<td>15</td>
<td>June 2012</td>
<td>Chemically polluted river water</td>
<td>28° 2’ 56” N</td>
<td>79° 29’ 23” E</td>
</tr>
</tbody>
</table>

Table 1: Collection sites of microalgal isolates from diverse habitats.
2.4. Identification of microalgal strains

2.4.1. PCR amplification, cloning and sequence analyses of 18S rDNA

For molecular confirmation of isolates, genomic DNA from the macerated biomass was extracted with a DNeasy plant mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer’s instructions. The extracted DNA was either used for polymerase chain reaction (PCR) or stored at −20 °C for further use. The genomic DNA containing 18S rDNA gene was amplified using two universal eukaryotic primers, namely (EUF: 5’-GTCAGAGGTGAAATTTCTTG GATTIA-3’ as the forward primer and EUR: 5’-AGGGCACAGCTAAT CAACG-3’ as the reverse primer), which are expected to amplify the ~700 bp region of 18S rDNA (Rasoul-Amini et al., 2009). The PCR reaction was performed in a total volume of 25 µL containing 2 µL of extracted DNA as template, 200 µM of each dNTPs, 1.5 mM MgCl₂, 0.5 µM forward & reverse primer and 0.5 U Taq DNA polymerase. The amplification was performed in an ABI Veriti thermo-cycler gradient with the PCR program conditions as described by Ghasemi et al. (2008). Similarly, the PCR reaction was performed using designed algae-specific primers, namely (FP1: 5′-GGCGGTAG TAAWWCTAGCTAATAC-3′ as the forward primer and RP1: 5′-AA AACGGCCGCTGTCACAAAGGC-3′) as the reverse primer with the following conditions for the PCR: 4 min initial denaturation at 95 °C; 35 cycles of 1 min denaturation at 95 °C, 1 min annealing at 56 °C and 2.5 min elongation at 72 °C; and a 10 min final elongation. The sequences of purified products (PCR purification kit, Qiagen, Germany) were determined using an ABI 310 automatic DNA sequencer (Applied Biosystems, California, and USA). The 18S rDNA sequences were compared with published sequences from the NCBI database to determine the nearest homologous sequences and the relative phylogenetic positions of individual isolates by ML tree analysis using MEGA ver. 6. In the present study, we mapped the existing universal primers from the literature as well as self-designed primers and evaluated them for their appropriateness for studying eukaryotic biodiversity.

2.4.2. Construction of degenerate algal-specific 18S rDNA primers

To confirm the results obtained using the universal primers, algae-specific degenerate primers were designed based on multiple sequence alignment of the 18S rDNA sequences using the software package BioEdit and Clustal W. The ribosomal encoded 18S rDNA gene sequences from a large data set corresponding to different genera of algae were obtained from NCBI. The specificity of the degenerate primer sets was chosen for amplifying a ~1.5–2.8 kb length, depending on the algal isolates. The specificity of these designed primers was also tested in silico using NCBI-BLAST to know the non-specific binding. The products of PCR (amplicons) were purified using a gel extraction kit (Qiagen, Hilden, Germany) and were cloned using the pGEM-T (Promega, USA) vector system following the manufacturer’s instructions. Colony-PCR was performed using universal M13 forward and reverse primers to confirm the positive clones. Two individual clones from each isolate were sequenced by Xcelris Genomics Pvt. Ltd (Ahmedabad, India). The NCBI-BLAST program was used to determine the sequences showing maximum homology.

2.5. Phylogenetic analysis

To ascertain the phylogeny of the isolated microalgae, their 18S rDNA sequences were aligned with the sequences of different closely related species that have been reported earlier. Multiple alignments were performed using Bio-Edit Sequence Alignment Editor ver. 7.2.5. The algorithms available in MEGA ver. 6, namely Maximum Likelihood (ML), Neighbour-Joining (NJ), and Maximum Parsimony (MP), were employed for constructing phylogenetic relationships among the algal isolates, and the reliability of the branches was assessed by bootstrapping the data with 1000 replicates.

2.6. Screening of microalgal isolates

Data on the growth curves of all the isolates were obtained during their cultivation. All the cultures with an initial cell count of 3 × 10⁶ cells/mL were cultivated under photoautotrophic conditions in a MC 1000-OD at 25 °C under 16 h of light (120 µE/m²/s) alternating with 8 h of darkness. The aeration rate was maintained at 0.12 mL/min. Microalgal growth was monitored by measuring the daily changes in OD at 680 nm with an OD viewer software attached to the cultivator. For studying growth kinetics, all isolates were cultivated in BBM media. The growth rate of microalgal isolates was estimated by fitting the OD at the exponential phase of each isolates to an exponential function in Eq. (1) (Wang et al., 2010).

\[
GR(\mu m) = \frac{(\ln OD_t - \ln OD_0)}{t}
\]

where OD₀ is the initial OD and ODₜ is the optical density measured on day t.

The microalgal cells were cultured for about 9 days on average and then cells were harvested, by centrifuging (10 min at 6000 rpm at 4 °C) followed by lyophilisation. The twenty two characterised isolates were further evaluated for their efficiency in producing biomass (dry biomass produced, expressed in milligrams per litre per day) and lipids (expressed as a percentage of total biomass on dry weight basis). The pellets were washed twice with sterile deionized water, lyophilized for 48 h, and their dry weight was determined gravimetrically.

2.7. Lipid extraction and preparation of FAME

Total lipids were extracted from the freeze-dried biomass following the method developed by Lewis et al. (2000) with some modifications: a known quantity (10 mg) of dried cell biomass was extracted in chloroform: methanol (2:1, v/v); the suspension was homogenised using vortex (Spinix, Maharashtra, India) for 2 min followed by centrifuging for 15 min at 10,000g. The extraction was repeated three times until the biomass became colourless. Method described by Christie and Han (2010) was employed to study FAME profiles. For preparation of FAME, 500 µL of toluene was added into dried lipid samples followed by 10 µL of internal standard (50 mg of C19:0-Methyl nonadecanoate), 400 µL of freshly prepared hydrogen chloride in methanol (prepared by adding 1 mL acetyl chloride drop wise to 10 mL of methanol on ice), 200 µL of antioxidant butylated hydroxy toluene (BHT) and incubated at 50 °C overnight. Following day, 1 mL of 5% NaCl and 1 mL of hexane was added to dried samples. Finally, solvent layer containing hexane were analysed using Gas chromatography.

2.8. Gas chromatography

The samples of FAMES were analysed by gas chromatography (GC) (Agilent 122-2332 column, Santa Clara, California, USA) with mass spectrometry (MS). Each sample (2 µL) was injected into a capillary column (DB-23: 30 mm × 0.25 mm; film thickness, 0.25 µm). The initial oven temperature of 70 °C was gradually increased by 10 °C/min until it reached 180 °C, which was maintained for 14 min. The oven temperature was raised again at 4 °C/min to reach 220 °C, which was maintained for 36 min. The detector temperature was set at 250 °C. The injector was maintained at 250 °C and a sample volume of 1 µL was injected. The carrier gas, namely helium, was released at 2.0 mL/min. A hexane injection was used as a blank. The sample injections used C19:0 (methyl nonadecanoate) as an internal standard to verify the
alignment of retention time with the calibration injection. The run time for a single sample was 36 min. Fatty acids were quantified by using the internal standard and identified by comparing the retention time with that of a known standard mixture of FAs (Supelco FAME mix C4-C24 (Bellefonte, Pennsylvania, USA). Fatty acid peaks were further analysed and integrated using a chemstation chromatography software (Agilent Technologies, Germany).

2.9. Evaluation of microalgal oil properties for biofuel

Some properties of the microalgal isolates were evaluated to screen them for biofuel production. A high cetane number (CN) of a FA denotes better combustion quality whereas the iodine value (IV) denotes the degree of unsaturation of the microalgal oil. A high IV indicates low oxidative stability of the fuel (Knotten. 2012). Hence, in the present study, CN, saponification value (SV), and IV were used in evaluating the FAME profiles using the following empirical Eqs. (2)–(4) proposed by Mandotra et al. (2014)

\[ \text{CN} = 46.3 + \frac{5458}{\text{SV}} - (0.225 \times \text{IV}) \quad (2) \]

\[ \text{IV} = \sum [(254 \times F \times D) / \text{MW}] \quad (3) \]

\[ \text{SV} = \sum [(560 \times F) / \text{MW}] \quad (4) \]

where, \( F \) is the percentage of each FA, \( D \) is the number of double bonds, and \( \text{MW} \) is the molecular weight of the FA. The standard biofuel properties as laid down in ASTM D6751 and EN 14214 were considered for evaluating the data.

2.10. Determination of microalgal carotenoids

Total carotenoids were obtained using the method described by García-Plazaola et al. (2012). In general, 25 mg of freeze-dried bio-mass was subjected to sonication at 40 kHz for 5 min at room temperature (Branson, Danbury, Connecticut, USA) to break the cell walls, and carotenoids and other compounds were extracted in acetone by continuous agitation for 3 min. The mixture of microalgal cells was then centrifuged at 4000 rpm for 15 min. The supernatant contained pigments, and the extraction process was repeated two more times so that the supernatant was colourless. All the extractions were carried out under darkness or under dim light to avoid photo-oxidation of the carotenoids. In the present study, we maintained all the isolates under uniform conditions to enable an accurate comparison of lipids and carotenoids productivities (expressed in milligrams per litre per day). The carotenoids

### Table 2

Molecular determination of microalgal isolates.

<table>
<thead>
<tr>
<th>Microalgal isolate</th>
<th>Designed primer amplicon product (kb)</th>
<th>Closest relative and GenBank accession No.</th>
<th>Universal primer amplicon product (bp)</th>
<th>Phylogenetic relative position using universal amplicon sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorella sp. (Q12)</td>
<td>1.53</td>
<td>Chlorella sp. ZB-2014 KJ734869 (99%)</td>
<td>669</td>
<td>Auxenochlorella sp. CCAP 211/61</td>
</tr>
<tr>
<td>Parachlorella kessleri (Y8)</td>
<td>1.77</td>
<td>Parachlorella kessleri strain SAG 211-11c KO2001114 (99%)</td>
<td>661</td>
<td>Chlorella sp. BUM11098</td>
</tr>
<tr>
<td>Chlorella sorokiniana (USA)</td>
<td>1.53</td>
<td>Chlorella sorokiniana strain UKM 2 KP262476 (99%)</td>
<td>350</td>
<td>Microctinium sp. TP-2008a CCAP 271/1</td>
</tr>
<tr>
<td>Chlorella sorokiniana (HS)</td>
<td>1.49</td>
<td>Chlorella sorokiniana strain SAG 211-31 KF673387 (99%)</td>
<td>337</td>
<td>Chlorella sp. ZB-2014</td>
</tr>
<tr>
<td>Auxenochlorella proteochlorocida (Goa)</td>
<td>1.53</td>
<td>Auxenochlorella proteochlorocida strain SAG 211-10a KO200150 (98%)</td>
<td>643</td>
<td>Auxenochlorella sp.</td>
</tr>
<tr>
<td>Scenedesmus sp. (V8)</td>
<td>2.06</td>
<td>Scenedesmus sp. KGU-D002 AB743827 (91%)</td>
<td>401</td>
<td>Eustigmatos vischeri CCAP 860/7</td>
</tr>
<tr>
<td>Scenedesmus sp. (Mn25)</td>
<td>2.81</td>
<td>Scenedesmus sp. KGU-D002 AB743827 (92%)</td>
<td>401</td>
<td>NI</td>
</tr>
<tr>
<td>Coelastrella sp. (Tn1)</td>
<td>2.81</td>
<td>Coelastrella striolata var. multistriata CCALA 309 XS13880 (91%)</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Coelastrella sp. (V3)</td>
<td>2.81</td>
<td>Coelastrella striolata var. multistriata strain CCALA; XS13880 (92%)</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Coelastrella sp. (P19)</td>
<td>1.93</td>
<td>Coelastrella striolata var. multistriata strain CCALA; XS13880 (98%)</td>
<td>342</td>
<td>Coelastrella striolata CAUP H 3602</td>
</tr>
<tr>
<td>Coelastrella sp. (Tree)</td>
<td>2.40</td>
<td>Coelastrella terestris strain CCALA 476JX513882 (92%)</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Scenedesmus sp. (Mn9)</td>
<td>2.81</td>
<td>Scenedesmus sp. NJ-1 JX286515 (93%)</td>
<td>353</td>
<td>Chlorella emersonii CCAP 211/8P</td>
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<tr>
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<td>Scenedesmus sp. KGU-D002 AB743827 (92%)</td>
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<td>Scenedesmus sp. (P58)</td>
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<td>Scenedesmus sp. KGU-D002 AB743827 (92%)</td>
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</tr>
<tr>
<td>Scenedesmus sp. (P115)</td>
<td>2.63</td>
<td>Scenedesmus sp. NJ-1 JX286515 (93%)</td>
<td>343</td>
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</tr>
<tr>
<td>Scenedesmus sp. (V11)</td>
<td>2.63</td>
<td>Scenedesmus sp. KGU-D002 AB743827 (92%)</td>
<td>658</td>
<td>Coelastrella sp. SAG 2471</td>
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<tr>
<td>Scenedesmus sp. (V9)</td>
<td>2.81</td>
<td>Scenedesmus sp. KGU-D002 AB743827 (92%)</td>
<td>656</td>
<td>Coelastrella striolata CAUP H 3602</td>
</tr>
<tr>
<td>Scenedesmus sp. (P152)</td>
<td>2.63</td>
<td>Scenedesmus sp. KGU-D002 AB743827 (89%)</td>
<td>343</td>
<td>Vischeria helvetica CCALA 514</td>
</tr>
<tr>
<td>Scenedesmus bijugus (Ladakh)</td>
<td>1.55</td>
<td>Scenedesmus bijugus var. obtusiusculus voucher ITZL 1830JK80696 (99%)</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Scenedesmus sp. (V15)</td>
<td>2.81</td>
<td>Scenedesmusaceae sp. Tow 9/21 P-14w AV197639 (93%)</td>
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<td>Pseurochrysos malhamensis DS</td>
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<td>Coelastrella sp. (P63)</td>
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<td>Coelastrella sp. BUM11113; KC218497 (95%)</td>
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<td>1.51</td>
<td>Vischeria stellata strain SAG 33.83 KF848919 (98%)</td>
<td>NI</td>
<td>Eustigmatos vischeri CCAP 860/7</td>
</tr>
</tbody>
</table>

NI: not identified. The species codes were defined based on the location from where they were collected. The isolates collected from Qatar were designated with code “Q”, from Hyderabad (HS), from St. louis “USA”, from Vishakapatnam “V”, from Delhi “P”, from Mangalore “Mn”, from Yamuna river “Y”, from Tamil nadu “Tn”, from Goa “Goa”, from Tree bark “Tree”, and from Jammu and Kashmir “Ladakh” respectively.
were analysed by injecting 10 μL of the carotenoid into an HPLC system (Shimadzu Co., Japan) equipped with a photodiode array detector. The pigments were separated using a reverse-phase C18 column coupled with a security guard column (Lune 3.0 mm C18 250 mm × 4.6 mm, 5 μm, Phenomenex, Torrance, California, USA). The binary mobile phase consisted of solvent A (acetonitrile: methanol, 7:1 v/v) and solvent B (acetonitrile:methanol:water: ethyl acetate, 7:0.96:0.04:2 v/v), and the following gradient method was used to separate the pigments, as recommended by García-Plazaola et al. (2012). The flow rate was set at 1.0 mL/min, and pigment content was detected by measuring the absorbance at 450 nm. The chromatographic peaks were analysed by comparing the retention time against the standard. The standards for lutein and canthaxanthin were bought from Sigma Chemical Co. (St. Louis, Missouri, USA); for astaxanthin and zeaxanthin, from Cayman Chemical Co.; and HPLC-grade acetonitrile, methanol, and ethyl acetate, from Fisher Scientific, USA.

3. Results and discussion

3.1. Isolation and identification of microalgae

The study aimed at to find species with the potential to produce a range of useful product, particularly carotenoids and biofuel. Two hundred samples were collected from aquatic and terrestrial rocky habitats from Asia, North America, and the Middle East (Table 1). Scanning electron microscopy imaging have been conducted in the selected species which showed the lipids and biofuel potential as depicted in Supplementary Fig. 1A–D. To further characterise, phylogenetic analysis using 18S rDNA sequences was performed.

3.2. Molecular identification of microalgal isolates

Confirming the true phylogeny of an organism is important because it can predict the adaptive traits developed through natural selection and their usefulness in biotechnology. In the present study, amplification of 18S rDNA sequence was carried out using both universal and self-designed primers. The amplification of 18S rDNA using universal eukaryotic primers showed efficient amplification of a unique single band varying in size from 341 bp to 669 bp (Table 2) in P155 (Scenedesmus sp.) from chemically polluted river water and in Q12 (Chlorella sp.) from a backwater. For accurate identification of the isolates, we designed new algae-specific degenerate primers based on the complete 18S rDNA sequences of widely different species of algae that belonged to a few representative species of Chlorophyta, Charophyta, Ochrophyta, Cryptophyta, Haptophyta, Rhodophyta, and Bacillariophyta. The newly designed primers aligned with and amplified more or less complete 18S rDNA sequences ranging from 1.5 kb to 2.8 kb depending on the algal strain. The identity index was 88% for the forward primer (GCCCGTAGTAAA WCTA SAGCTAATAC) and did not amplify up in any other environmental cultures except algae, whereas that for the reverse primer (AAAAACGGGCGGTGTGTA CAAAGGGC) was 92%; however, that primer also detected another class of higher-order organisms. We tested the specificity of the primers using different samples of algae from the environment, which were PCR-amplified, cloned, and sequenced. In none of the clones, DNA sequences from other microbes from higher-order organisms highlighted; these primer combinations were considered useful as molecular tools for specific amplification of 18S rDNA gene of algal species alone. The primers used to amplify the rDNA region successfully amplified DNA from all the twenty
two microalgae isolates. The PCR products fell into two size categories, namely 1.491–1.938 kb and 2.4–2.8 kb. The amplified and cloned 18S rDNA sequences using degenerate algae-specific primers were sequenced to obtain detailed information on their close relatives through GenBank accessions number (Table 2). Although we tried to analyse all the sequences together, due to the differences in size, no common sequences were found for pair-wise analysis and for multiple sequence alignments. The phylogenetic positions of the 18S rDNA sequences in the size category 1.491–1.938 kb were analysed separately along with other close relatives of algal species obtained through NCBI-BLAST (Fig. 1A and B). Percentage similarity values obtained after pair-wise alignment of the rDNA sequences of the isolates in that size category are listed in Table 2. The relative phylogenetic positions based on the designed primers sequence were matched with the results of morphological studies and served to further confirm the identification of the isolates. Other unique intronic positions were observed in the isolates of Scenedesmus sp. collected from diverse habitats. These sequences were compared using the software Bio-edit to define the novel pattern of intronic that aligned with known Scenedesmus sp. reported earlier (Fig. 1C). Although the use of universal primers allows direct comparison between studies, in analysing...
16h Culture grown with 16 h light and air.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Growth rate (OD, mu)</th>
<th>Biomass productivity (mg L^{-1} d^{-1})</th>
<th>Lipid productivity (mg L^{-1} d^{-1})</th>
<th>Lipid content (% dry wt.)</th>
<th>Astaxanthin productivity (mg L^{-1} d^{-1})</th>
<th>Lutein content (mg/g)</th>
<th>Lutein productivity (mg L^{-1} d^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlorella</em> sp. (Q12)</td>
<td>0.40</td>
<td>169.81 ± 5.22</td>
<td>39.72 ± 3.89</td>
<td>24.97 ± 2.69</td>
<td>0.18</td>
<td>2.26</td>
<td>0.36</td>
</tr>
<tr>
<td>Parachlorella kessleri (Y8)</td>
<td>0.39</td>
<td>159.49 ± 7.63</td>
<td>31.71 ± 4.34</td>
<td>21.42 ± 3.19</td>
<td>0.07</td>
<td>0.28</td>
<td>0.04</td>
</tr>
<tr>
<td><em>Chlorella</em> sorokiniana (USA)</td>
<td>0.32</td>
<td>134.94 ± 12.52</td>
<td>31.94 ± 1.26</td>
<td>28.58 ± 1.33</td>
<td>n.d.</td>
<td>0.68</td>
<td>0.07</td>
</tr>
<tr>
<td><em>Chlorella</em> sorokiniana (HS)</td>
<td>0.27</td>
<td>102.26 ± 9.65</td>
<td>11.51 ± 2.61</td>
<td>13.55 ± 2.77</td>
<td>0.06</td>
<td>5.90</td>
<td>0.5</td>
</tr>
<tr>
<td>Auxenochlorella protothecoides (Goa)</td>
<td>0.53</td>
<td>167.11 ± 12.22</td>
<td>31.33 ± 3.64</td>
<td>24.97 ± 2.69</td>
<td>0.06</td>
<td>0.76</td>
<td>0.11</td>
</tr>
<tr>
<td>AG, 12h Culture grown with agitation and 12 h light.</td>
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<tr>
<td>AG, 18h Culture grown with agitation and 18 h light.</td>
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<tr>
<td>24h Culture grown with 24 h light and air.</td>
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</table>

Data calculated at average of nine days; n.d. not detectable. Experiments were performed in triplicates and data for biomass and lipid productivities are expressed as mean ± SD.

Table 4

Comparison of lipid productivity of microalgal isolates with other published data.

<table>
<thead>
<tr>
<th>Microalgal isolates</th>
<th>Lipid productivity (mg L^{-1} d^{-1})</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlorella</em> sp.(Q12)</td>
<td>39.72 ± 3.89</td>
<td>This study^{10th}</td>
</tr>
<tr>
<td><em>Chlorella</em> SDEC-6</td>
<td>9.6</td>
<td>Song et al.(2014)^{24th}</td>
</tr>
<tr>
<td><em>Chlorella</em> sp.</td>
<td>83.3</td>
<td>Chuirrip and Torpee (2012)^{16th}</td>
</tr>
<tr>
<td><em>Scenedesmus</em> sp. (PS8)</td>
<td>43.81 ± 5.15</td>
<td>This study^{10th}</td>
</tr>
<tr>
<td><em>Scenedesmus</em> bijugus</td>
<td>40.14 ± 3.31</td>
<td>This study^{10th}</td>
</tr>
<tr>
<td><em>Scenedesmus</em> sp. (PVS2)</td>
<td>8.22</td>
<td>Shan et al.(2015)</td>
</tr>
<tr>
<td><em>Scenedesmus</em> bijugus</td>
<td>21.5</td>
<td>Tripathi et al.(2015)^{AG.16th} and P. bidentata</td>
</tr>
<tr>
<td><em>Scenedesmus</em> sp. (P63)</td>
<td>13.52</td>
<td>Song et al.(2014)^{AG.16th}</td>
</tr>
<tr>
<td><em>Coelastrella</em> sp.</td>
<td>43.55 ± 6.28</td>
<td>Lutein study^{10th}</td>
</tr>
<tr>
<td><em>Vischeria</em> Stellata</td>
<td>13.9</td>
<td>Karpagam et al.(2015)^{AG.12th}</td>
</tr>
</tbody>
</table>

^{10th}Culture grown with 16 h light and air.

^{24th}Culture grown with 24 h light and air.

^{AG.16th}Culture grown with agitation and 18 h light.

^{AG.12th}Culture grown with agitation and 12 h light.

N and P limitation: Cultures supplemented with low nitrogen and phosphorus concentrations.

Environmental samples, such use places some limitations on the interpretation of results. These limitations can be overcome by obtaining whole sequences from 18S rRNA genes as reference trees. Since the selection of primers has a significant impact on the results obtained from environmentally diverse ecosystems, such reference trees could be used for studying large algal populations. The present study thus provided a designed primer pair more suitable as a molecular marker in studying eukaryotic diversity or in characterising microalgae. However, the overall morphological features and 18S rDNA sequences confirm the taxonomical position of these isolates to great extent.

3.3. Comparative analysis of photo-autotrophic growth, biomass, lipids, and FAME profile of microalgal isolates

Rapid growth is an important prerequisite to obtaining a high cell density, intracellular lipids, under optimal culture conditions (Talebi et al., 2013). Under identical conditions, the isolates varied a great deal in their ability to produce biomass: the productivity ranged from 83.33 ± 8.51 mg L^{-1} d^{-1} to 174.77 ± 6.75 mg L^{-1} d^{-1} (Fig. 2). Microalgal growth as assessed by OD in the isolates drawn from four different habitats, namely freshwater, chemically polluted river water, backwater, and land, is shown in (Fig. 2A–D). The average specific growth rate was estimated at the exponential growth phase (Table 3). The growth varied among the isolates in the same medium (BBM) under similar culture conditions. In the present study, lipid productivity and biomass productivity – expressed as daily production, in milligrams, of biomass or lipids – were considered equally important in selecting appropriate strains for biofuel production.
Under experimental conditions, total lipid content of freshwater isolates was highly variable (13.15 ± 1.87–36.52 ± 1.81% of the dry weight) whereas that of backwater isolates peaked at 36.53 ± 2.13% and that of land isolates was average 15.83 ± 1.65 (Table 3). Lipid productivity varied between 10.05 ± 1.98 mg L\(^{-1}\) d\(^{-1}\) and 43.81 ± 5.15 mg L\(^{-1}\) d\(^{-1}\). Of all the Scenedesmus isolates studied, one from chemically polluted river water (P58) and one from freshwater (Ladakh), showed higher productivity 43.81 ± 5.15 mg L\(^{-1}\) d\(^{-1}\) and 40.14 ± 3.31 mg L\(^{-1}\) d\(^{-1}\) for lipids and 155.71 ± 14.34 mg L\(^{-1}\) d\(^{-1}\) and 174.77 ± 6.75 mg L\(^{-1}\) d\(^{-1}\) for biomass, respectively (Table 3) – than that reported elsewhere, such as (1) Scenedesmus sp. (Tripathi et al., 2015), and Scenedesmus SDEC-8 (Song et al., 2014) under non-aerated conditions. Chlorella sp. (Q12) showed higher productivity (39.72 ± 3.89 mg L\(^{-1}\) d\(^{-1}\) for lipids and 169.81 ± 5.22 mg L\(^{-1}\) d\(^{-1}\) for biomass) among previously reported Chlorella sp., including one reported by Cheirsilp and Torpee (2012), Chlorella sp. SDEC-6 (Song et al., 2014) as shown in Table 4. The isolate from high altitudes, namely Scenedesmus bijugus (Ladakh), also produced more biomass (174.77 ± 6.75 mg L\(^{-1}\) d\(^{-1}\) and lipids (40.14 ± 3.31 mg L\(^{-1}\) d\(^{-1}\)) than the figures reported for S. bijugus grown on wastewater effluent diluted with BG11 medium (Shin et al., 2015). The other group of isolates including Coelastrella showed high biomass and lipid productivity. Coelastrella sp. (P63) from chemically polluted river water produced 152.93 ± 13.35 mg L\(^{-1}\) d\(^{-1}\) of biomass and 43.55 ± 6.28 mg L\(^{-1}\) d\(^{-1}\) of lipids – figures that were comparable to those reported by others, such as Coelastrella M 60 (Karpagam et al., 2015) (Table 4). From above results, microalgal isolates from highly chemically polluted as well as freshwater habitats demonstrated the potential to produce large quantities of lipids and biomass. Scenedesmus sp. (P58), S. bijugus (from Ladakh), Coelastrella sp. (P63), and Chlorella sorokiniana (USA) were the most promising lipid and biomass producers among all the twenty two isolates (marked by an asterisk in Fig. 2).

Besides lipid productivity, FA profiles of the isolates were further examined and compared because FA composition and the types of FAs produced are considered important for the quality of biodiesel. The quality depends mainly on the unsaturation ratio because unsaturated fatty acids (UFA) enhance cold-flow properties whereas saturated FAs maintain good oxidative stability (Hoekman et al., 2012). All the examined and compared because FA composition and the components for biofuel since it gives a good balance between cold flow property and oxidative stability (Hoekman et al., 2012). All the above four isolates showed a good biofuel characteristics. In the present study, Parachlorella kessleri was the only isolate that produced large amounts of C 16:0 (up to 59.1%). Microalga rich in MUFA (particularly, palmitoleic acid (16:1) and oleic acid (18:1) (Hoekman et al., 2012) and SFAs are good for biodiesel production (Stansell et al., 2012).

To ascertain the suitability of the isolates as producers of biofuel, a few other properties of the lipids, namely CN, IV, and SV, were analysed based on FAME profiling. According to the EN14214 standard, SV and CN should be neither higher than 120 g I\(_2\)/100 g nor lower than 47 g I\(_2\)/100 g (Sun et al., 2014). Six isolates in the present study met the CN norms specified in USA (ASTM D6751) and twelve, those in Europe (EN 14214) (Fig. 4). Four isolates failed to meet any of the above specifications; however, they be important because they have a high (>13%) proportion of C18:3 acids. In the present study, IV for Chlorella sp. (between 12 g I\(_2\)/100 g and 103 g I\(_2\)/100 g) was consistent with that reported for Chlorella sp. by Wu and Miao (2014). Thus, many of the isolates among the twenty two were suitable for biofuel production and the rest had other valuable properties (Table 5). The high content of SFAs and oleic acid in a few of the microalgae isolated in the present study shows their potential for biofuel production.

### 3.4. Lutein and astaxanthin productivity

It is vital to select isolates that have the potential to produce large quantities of the targeted products, lipids and carotenoids if their use is to be commercially cost effective. The ability of the isolates to produce lutein and astaxanthin was also assessed. The major carotenoid was lutein, followed by astaxanthin. The isolates were grown autotrophically and without any known stress in MC 1000-OD and their production of intracellular lutein and
astaxanthin as well as of biomass was recorded at average of nine days (Table 3). *Chlorella* sp. (Q12), the most productive isolate in the present study, produced more biomass, lutein, and astaxanthin. *S. bijugus* (Ladakh), the second most productive isolate, was comparable to *Scenedesmus* sp. (Yen et al., 2011). Sánchez et al. (2008) claimed that *Scenedesmus almeriensis* are the best candidates with high lutein content. Two other isolates, namely Coelastrella sp. P63 and V3, were also highly productive. Whereas the isolates (V3) in the present study recorded the highest lutein content (up to 6.49 mg/g) with high lutein productivity (0.81 mg L⁻¹ d⁻¹). The present study shows that a few of the isolates demonstrated the potential to produce carotenoids as well as lipids, a potential that could be exploited for obtaining multiple products.

### 4. Conclusions

The overall results from the present study confirm that some of the isolates *S. bijugus*, *Coelastrella* sp., *Chlorella* sp., and *Aurenoclorella protothecoides* showed the potential to produce significant quantities of multiple products. The designed primer sets provided better insights into biodiversity and helped in finding new species that would have been hard to find through microscopic observations alone. *S. bijugus* was the most productive among the four isolates (V3) in the present study recorded the highest lutein content (up to 6.49 mg/g) with high lutein productivity (0.81 mg L⁻¹ d⁻¹). The present study shows that a few of the isolates demonstrated the potential to produce carotenoids as well as lipids, a potential that could be exploited for obtaining multiple products.
promising microalgal isolates selected. To the best of our knowl-
edge this is the first report of a microalgal isolate with multiple
potential. Future studies can further maximise metabolites produc-
tivity potential.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in
the online version, at http://dx.doi.org/10.1016/j.biortech.2016.03.
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